The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Characterisation of KPC, heterozygously and homozygously *Zeb1* depleted KPC tumours.

(a) Representative Zeb1-immunolabeling of a GFP lineage-traced primary tumour showing Zeb1/GFP double-positive tumour cells (arrows). n=5 independent tumors. Scale bar, 50 µm. **(b)** Representative consecutive sections of HE and indicated immunohistochemical stainings of four Zeb1 expressing KPC tumours demonstrating the heterogeneity in phenotype, grading and marker expression. A representative differentiated Zeb1-negative KPCZ tumour is shown for comparison. Arrows indicate Zeb1 positive tumour cells in the differentiated KPC tumour. n= 15 KPC, 13 KPCZ independent tumours. Scale bar, 100 µm. **(c)** Tumour-free survival of KPC mice vs. KPC mice with a heterozygous deletion of *Zeb1* (KPCz) (n= 15 KPC, 16 KPCz independent tumours); log-rank (Mantel-Cox) test); tumour volume (0 = start of MRI measurements, n=12 KPC, 14 KPCz independent tumours); error bars show mean ±S.E.M.; multiple t-tests with correction for multiple comparison using the Holm-Sidak method; grading, local invasion and relative ECM deposition of the respective tumours (n=31 KPC, 17 KPCz; Mann-Whitney test (two-tailed); percentage of metastasized tumours (n=35 KPC, 17 KPCz independent tumours; Chi-square test (two-tailed); n.s. = not significant.

Supplementary Figure 2: Characterisation of KPC vs. KPCZ tumours.

Representative images of immunohistochemical and histological stainings of KPC and KPCZ tumours and quantifications of the indicated markers are given. Asterisks label Zeb1-expressing stroma cells in KPCZ tumours. Specific blue MTS staining labels collagen fibres. Scale bars, 100 µm, for lower left image 50 µm. n=48 KPC, 29 KPCZ independent tumours for Zeb1 and MTS; n= 15 independent tumours for KPC, 13 independent tumours for KPCZ for all other markers, error bars show mean ±S.D.; ****p<0.0001, n.s. = not significant, Chi-square test (two-tailed) for Zeb1, E-cadherin and Sox2, unpaired Student's t-test (two-tailed) for Ki67 and Casp3 (with Welch's correction), Mann-Whitney test (two-tailed) for ECM and CD31.

Supplementary Figure 3: Characterisation of differentiaton markers in KPC vs. KPCZ tumours.

(a) Representative images of positive and negative immunohistochemical stainings and statistical analysis for the indicated EMT-TFs. Scale bar, 150 µm. n= 14 independent tumours for KPC, 13 independent tumours for KPCZ, Chi-square test (two-tailed); n.s. = not significant. **(b)** Representative images of immunohistochemical stainings and statistical analysis for expression of Gata6. Scale bar, 150 µm. n=14 independent tumours for KPC, 13 independent tumours for KPCZ; error bars show mean ±S.D.; Mann-Whitney test (two-tailed), ***p<0.001. **(c)** Representative images of differentiated KPCZ and undifferentiated KPC primary tumours (PT) and corresponding metastases (Met) with the same phenotype. Immunohistochemical labelling of Zeb1 expressing tumour cells in the KPC PT and Met (arrows). L= liver or lung tissue. n= 19 KPC, 4 KPCZ independent tumours and corresponding metastases. Scale bar, 100 µm.

Supplementary Figure 4: Characterisation of KPC vs. KPCZ tumour derived cell lines.

(a) Bright field image of primary cell lines from KPC and KPCZ tumours as well as HE stainings of the respective tumours after grafting in syngeneic mice and of the respective primary tumours are shown. Scale bars, 100 µm for bright field, 75 µm for HE stainings. **(b)** MTT viability assay for the isolated tumour cell lines after treatment with the indicated doses of gemcitabine and erlotinib. The calculated IC50 values for gemcitabine are shown. n=3 biologically independent experiments, error bars show mean ±S.E.M. **(c)** Tumour onset after subcutaneous injection of 1 x 10^5 KPC and KPCZ cells into syngeneic mice. n=4 mice/cell line, error bars show mean ±S.E.M. **(d)** Tumour grading, grading at invasive regions and relative ECM deposition of one representative tumour/cell line analysed in c) (n=6 tumours for KPC, n=5 tumours for KPCZ); error bars show mean ±S.D.; *p<0.05, **p<0.01, Mann-Whitney test (two-tailed).

Supplementary Figure 5: Depletion of *Zeb1* affects tumour promoting capacities.

(a) Representative images of one visual field (n=6 fields/cell line) showing GFP+ cells/cell clusters in the lungs (green dots) 2 h after i.v. injection of KPC and KPCZ tumour cells and control lungs. Scale bar, 500 µm. **(b)** No. of tumours after subcutaneous injection of the indicated cell numbers for the KPC and KPCZ tumour cell lines and calculated fraction of tumourigenic cells. inf =infinite, Chi-square test. **(c)** Representative images showing spheres of KPC and KPCZ tumour cells. Scale bar, 500 µm and 50 µm for higher magnifications. **(d)** Percentage of cells in KPC and KPCZ lines

positive for the indicated markers or marker combinations; n=2 biologically independent experiments, error bars show ±S.D. Source data see Supplementary Table 5, Statistics Source Data. Relative mRNA expression levels (qRT-PCR) of indicated genes, mRNA levels of KPC661 was set to 1; n=3 biologically independent experiments, Mann-Whitney test (two-tailed), *p<0.05, **p<0.01, error bars show mean ±S.E.M.

Supplementary Figure 6: Depletion of *Zeb1* reduces early PanIN lesions.
(a) Consecutive sections showing representative HE and PAS stainings of precancerous PanIN lesions in the pancreas of two different 6 month old KC and of one KCZ mice. Specific dark blue PAS staining indicates the mucin-rich PanIN lesions. Scale bars, 2.5 mm and 150 µm for higher magnifications. Quantification of the PanIN area (% of pancreas area).n=12 KC and 7 KCZ independent mice , error bars show mean ±S.D.; **p<0.01, unpaired Student's t-test (two tailed) with Welch's correction. **(b)** Gene set enrichment analyses (GSEA) of transcriptome data from KPCZ vs. KPC cells reveals reduction of gene signatures associated with cancer mesenchymal transition and Zeb1 targets in KPCZ vs. KPC cell lines. NES = normalized enrichment score; FDR=false discovery rate.

Supplementary Figure 7: Depletion of *Zeb1* reduces tumour cell plasticity.
(a) Relative mRNA expression levels (qRT-PCR) of indicated genes in KPC and KPCZ cell lines treated for different times with TGFβ (time points: 0, 6 h, 1, 3, 7, 14, 21 days). mRNA levels of cell line 661 at day 0 were set to 1. n=3 biologically independent experiments, error bars show mean ±S.E.M. Statistical analysis is shown for the comparison of TGFβ treated to untreated samples (grey bars) of each individual cell line *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, unpaired Student's t-test (one-tailed) Source data see Supplementary Table 5, Statistics Source Data. **(b)** Table showing log2FC in mRNA expression levels (microarray) of genes previously determined as common ZEB1/YAP targets in KPC and KPCZ cell lines upon TGFβ treatment for 14 days. (cut-off: adj. p-value<0.05 and $\log_2$FC>0.5). **(c)** Representative images of consecutive sections of immunohistochemistry for Ck19 and Zeb1 comparing the plasticity of Zeb1 expression in central and invasive tumour regions. Tumours derived from one KPC and one KPCZ cell line are shown. Asterisks label Zeb1 expression in stroma cells, arrows indicate Zeb1 expression in tumour cells at the invasive front. Ck19

expression is shown to identify cancer cells. n= 15 KPC, 13 KPCZ independent tumours, Scale bars, 50 μm and 150 μm for higher magnifications.

Supplementary Figure 8: Unprocessed scans of immunoblots

SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1: Overview of all KPC and KPCZ mice included in the study

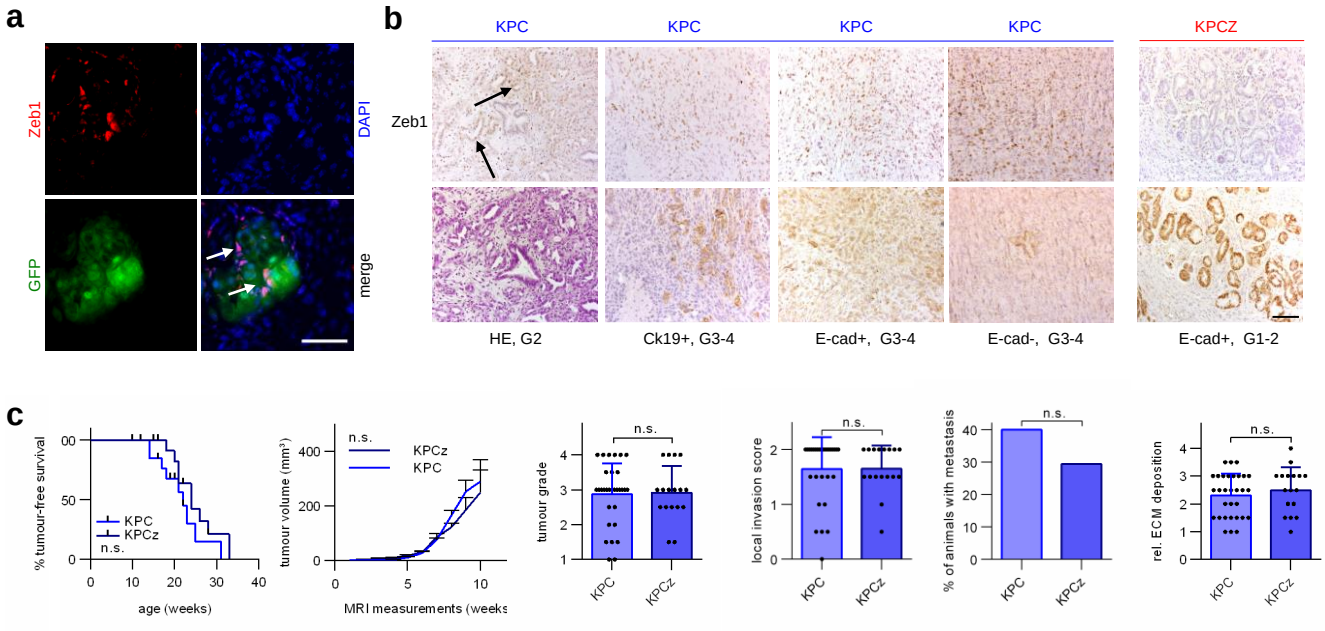
Supplementary Table 2: Genes up- or downregulated upon long-term TGFβ treatment in epithelial KPC and KPCZ cells

Supplementary Table 3: Information on primers used for qRT-PCR

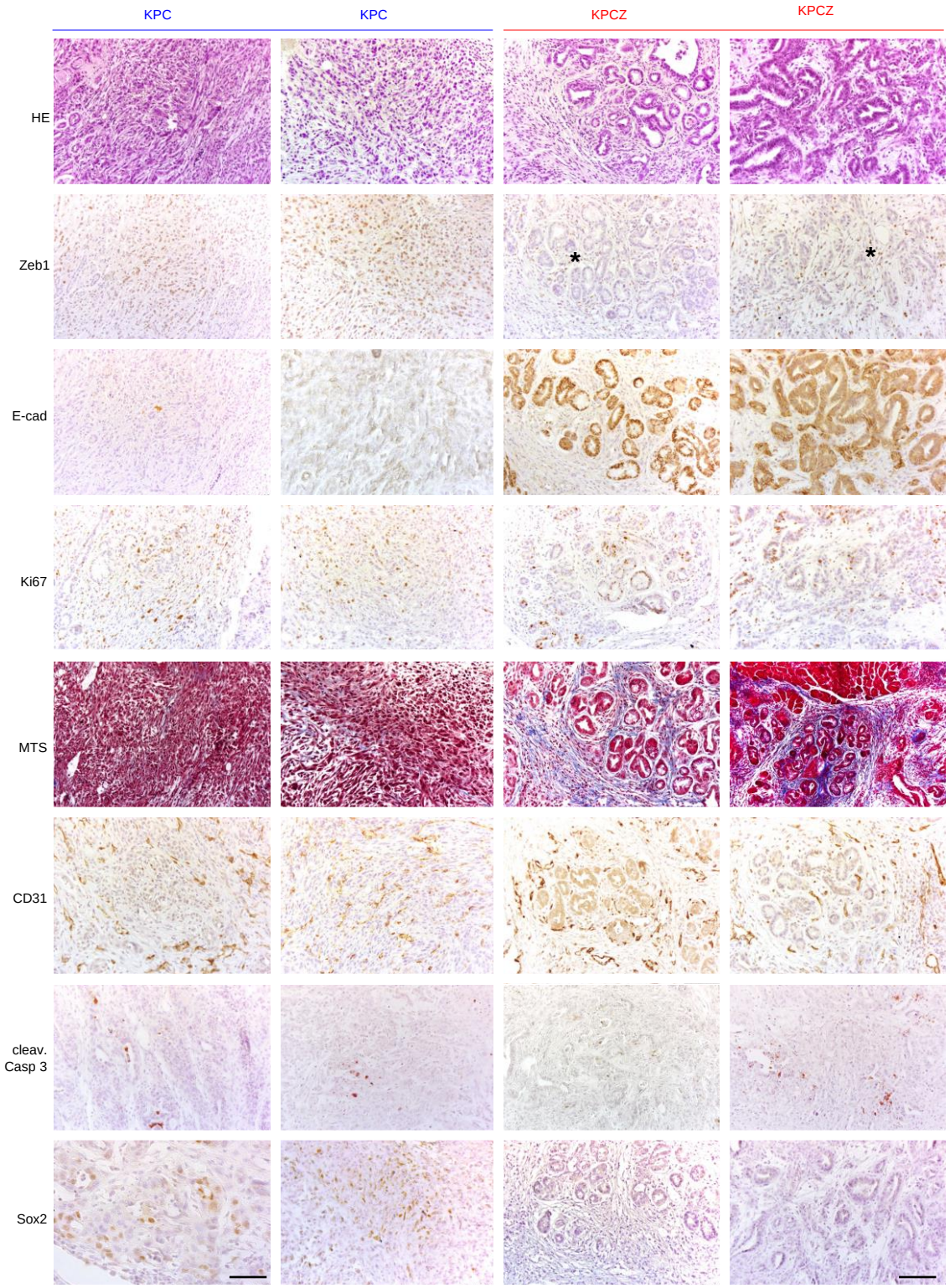
Supplementary Table 4: Selected 36 gene sets used for gene set enrichment analysis.

Names and online link for the 36 publically available gene sets used for gene set enrichment analysis related to pancreatic cancer, Zeb1 or metastasis.

Supplementary Table 5: Statistics Source Data



Krebs, Supplementary Fig. 1

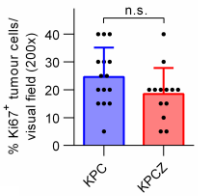


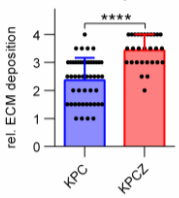
Zeb1⁺ tumour cells	KPC	KPCZ
no	13	29
yes	35	0
%	72.9	0.0

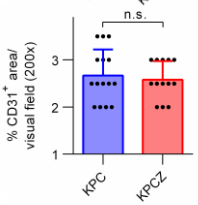
p<0.0001

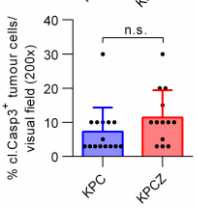
Membranous E-cadherin staining	KPC	KPCZ
no	7	0
yes	8	13
%	53.3	100.0

p<0.01







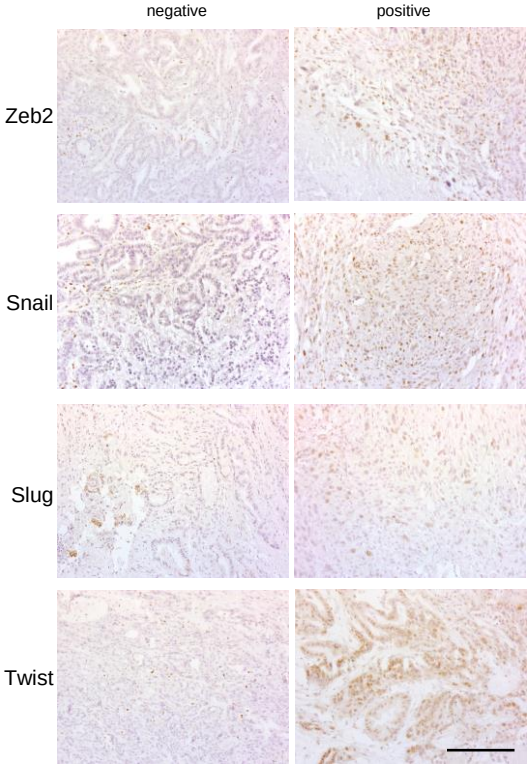


Sox2⁺ tumour cells	KPC	KPCZ
no	6	12
yes	9	1
%	60.0	7.69

p<0.01

Krebs, Supplementary Fig. 2

a



Zeb2⁺ tumour cells	KPC	KPCZ
no	8	13
yes	6	0
%	42.8	0.0

p<0.01

Snail⁺ tumour cells	KPC	KPCZ
no	7	10
yes	7	3
%	50.0	23.1

n.s.

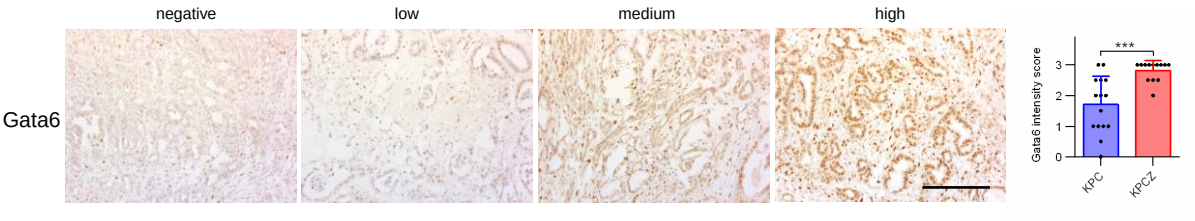
Slug⁺ tumour cells	KPC	KPCZ
no	11	13
yes	3	0
%	21.4	0.0

p=0.07

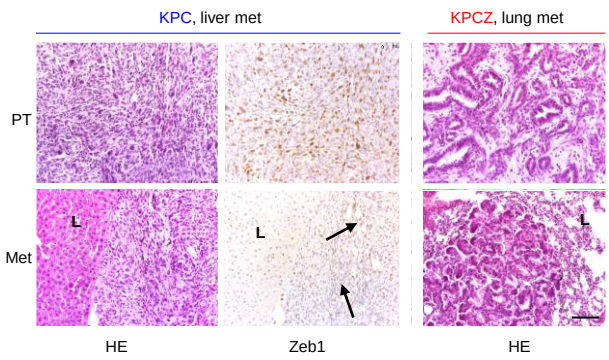
Twist⁺ tumour cells	KPC	KPCZ
no	3	3
yes	11	10
%	78.6	76.9

n.s.

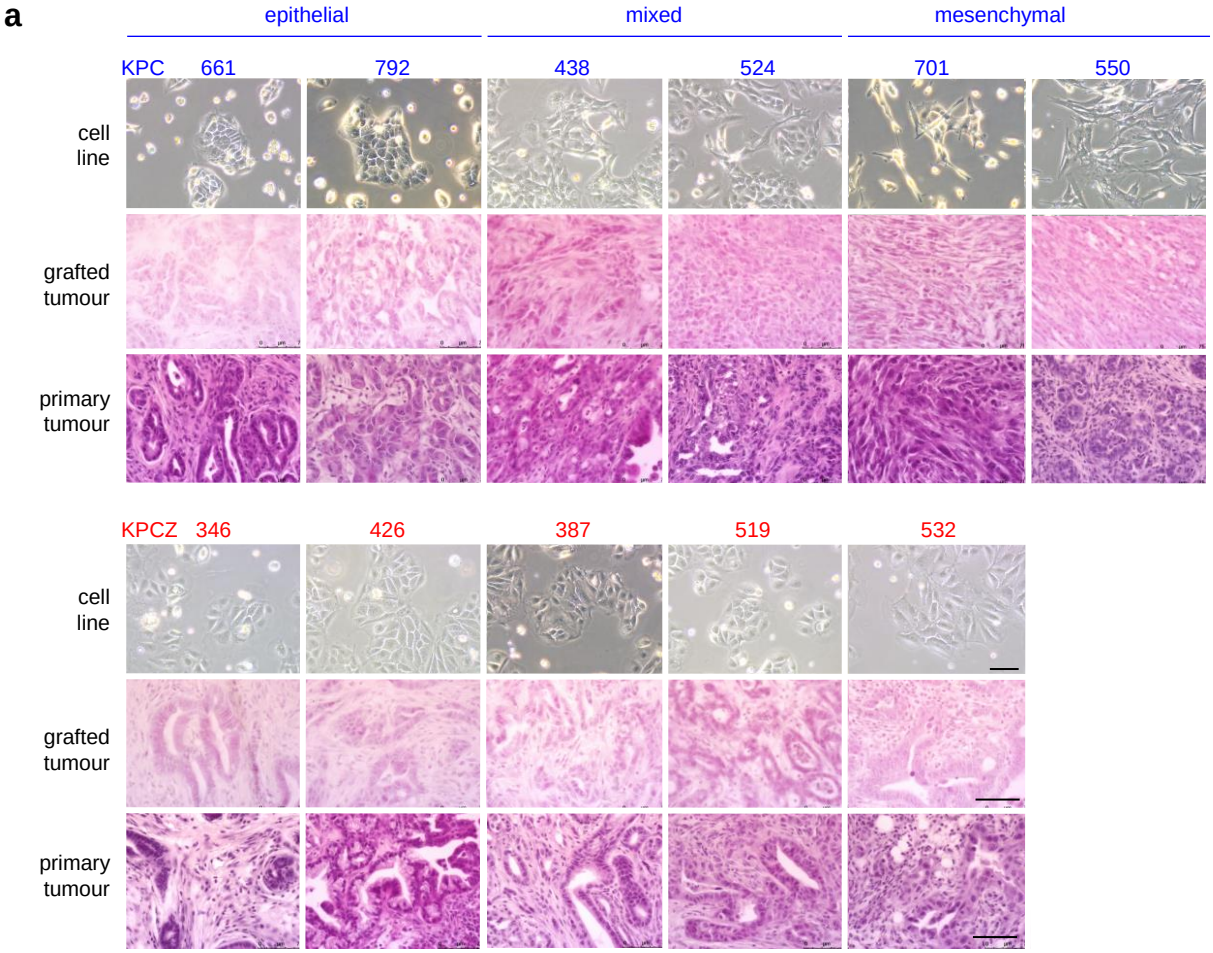
b

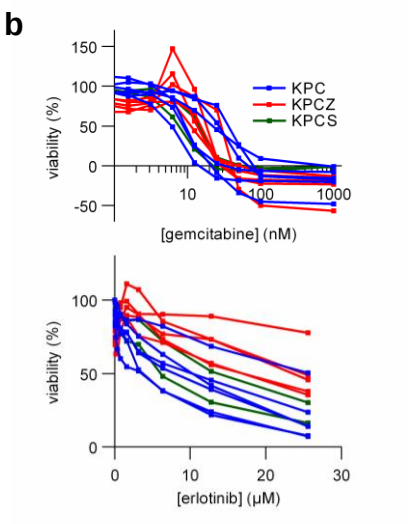


c

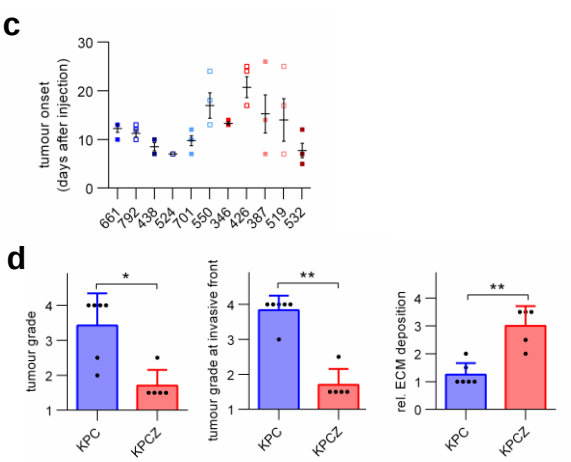


Krebs, Supplementary Fig. 3



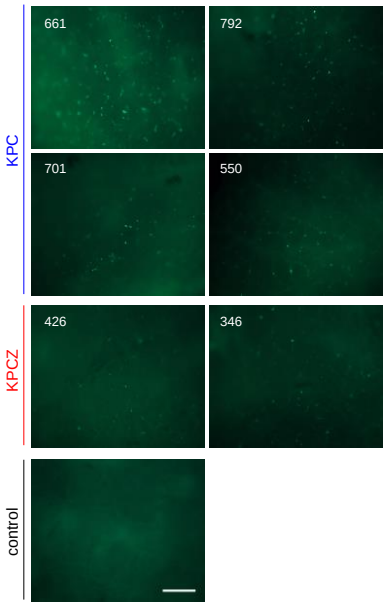


Cell line	IC50 (nM)
661	43.84
792	26.65
438	6.71
524	33.74
701	9.76
550	14.22
346	14.28
426	46.09
387	20.48
519	32.24
532	19.97
2265	7.422
2529	15.05



Krebs, Supplementary Fig. 4

a

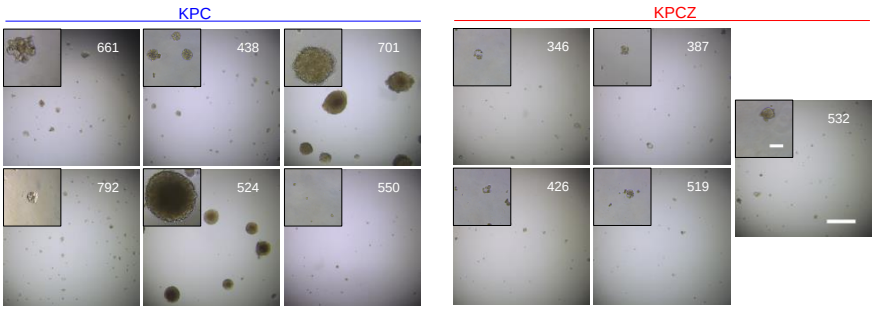


b

	500 cells	2500 cells	12500 cells	Stem cell fraction
661	1/4	3/4	4/4	1/ 1773
792	1/4	3/4	7/8	1/ 4012
438	2/4	0/4	4/4	1/ 4300
524	3/4	2/4	6/8	1/ 5210
550	0/4	1/4	3/6	1/16143
701	0/4	2/4	1/4	1/17484
532	0/4	2/4	3/4	1/17700
346	0/4	0/4	2/4	1/24215
426	0/4	0/4	1/4	1/55516
519	0/4	0/4	1/4	1/55516
387	0/4	0/4	0/4	Inf

all KPC vs KPCZ cell lines p < 0.0001 (****)

c



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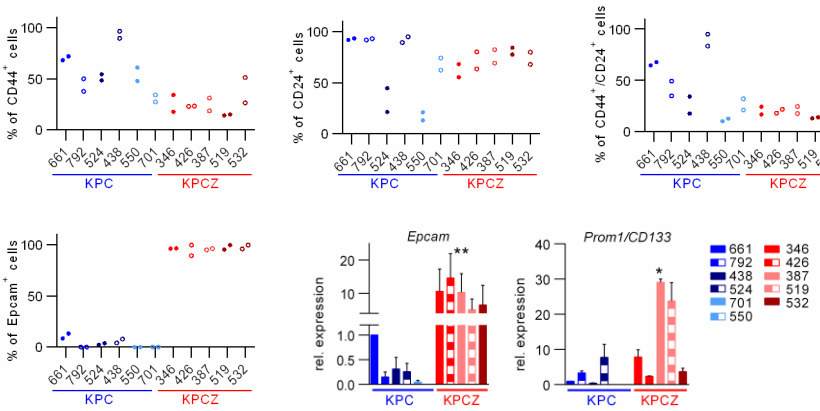

Krebs, Supplementary Fig. 5

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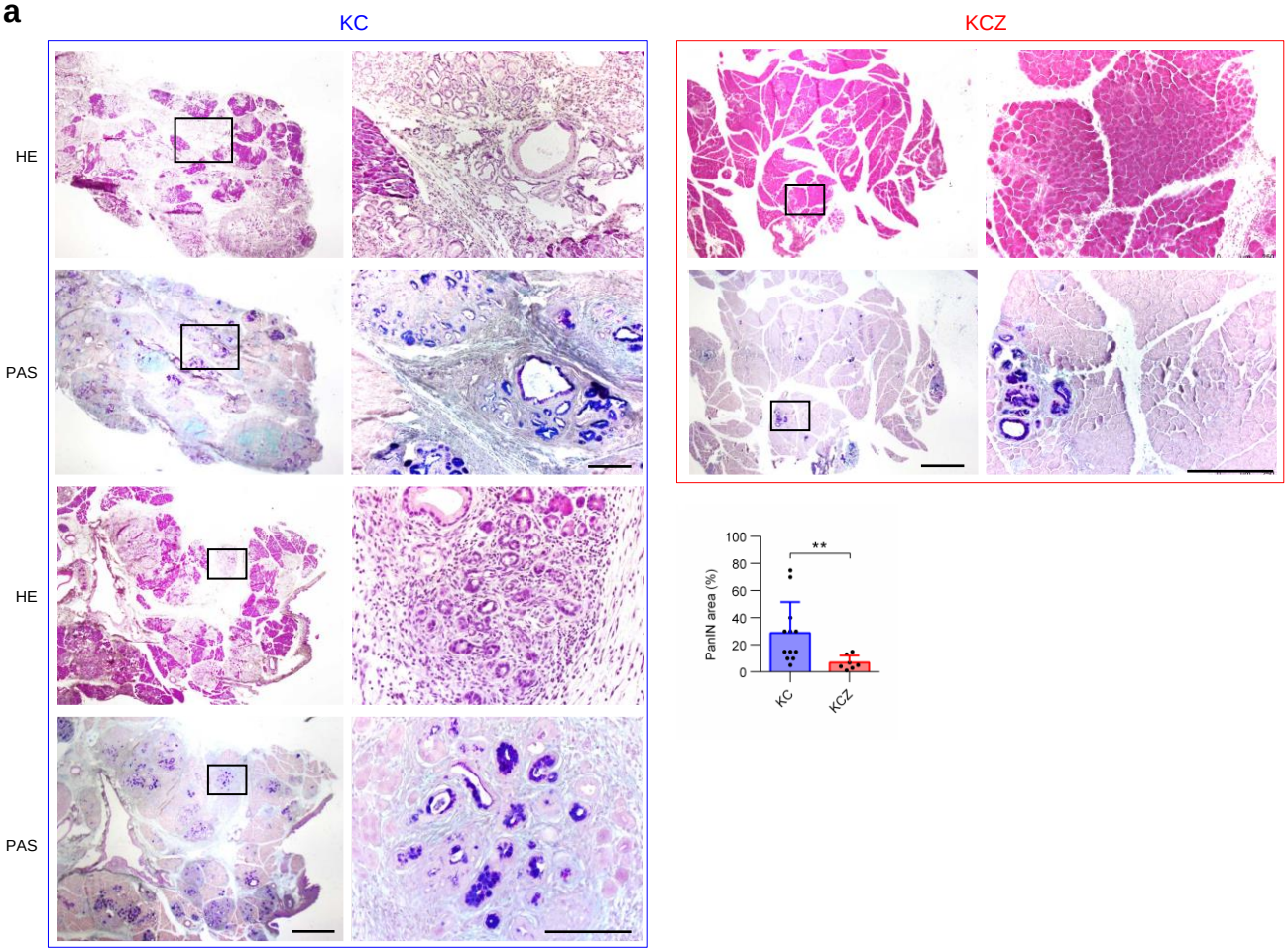

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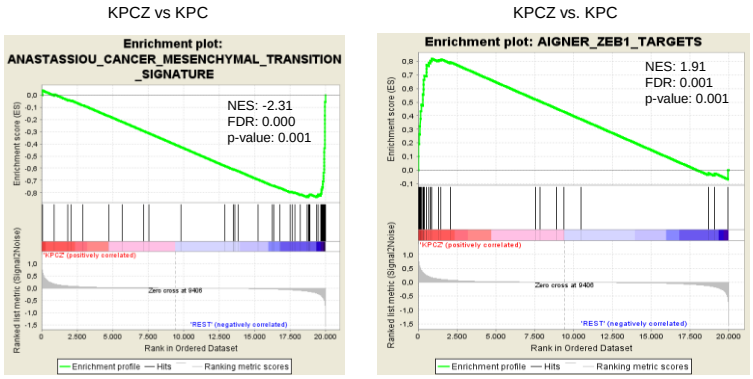
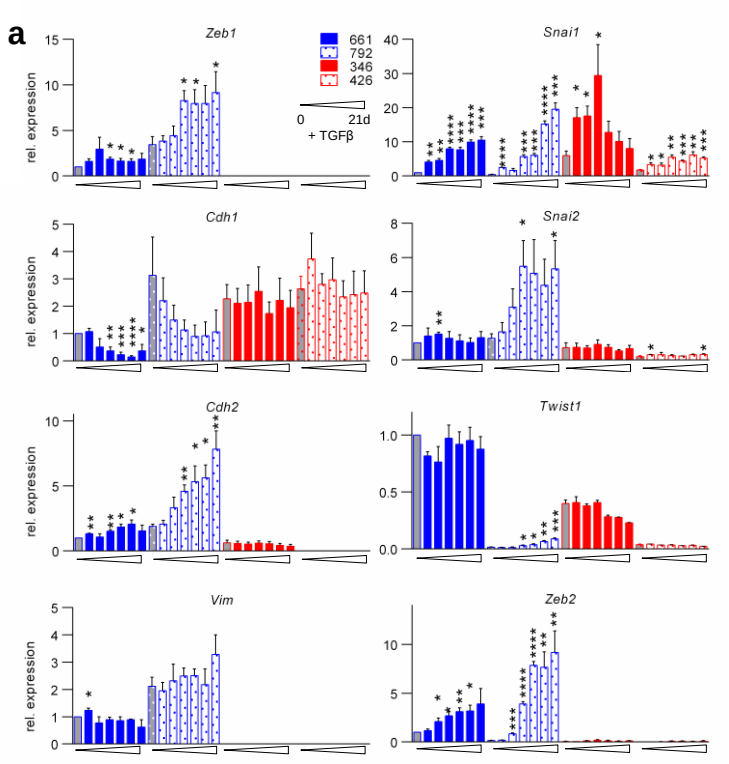


Krebs, Supplementary Fig. 6

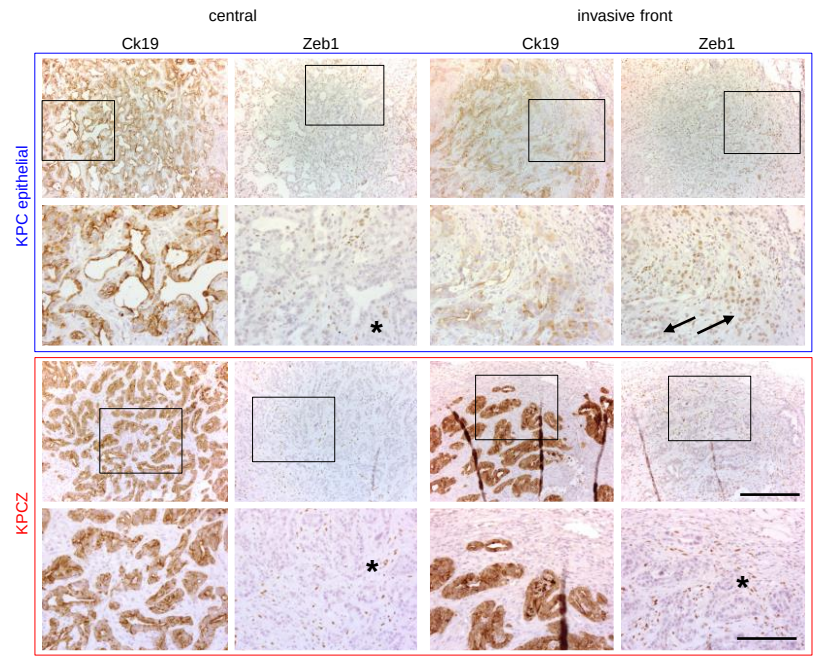
a



b

log2FC	661+TGFβ vs 661	792+TGFβ vs. 792	346+TGFβ vs 346	426+TGFβ vs 426
Dusp1	1.2*	3.3*	-0.3	-0.2
Lhfp	1.5*	1.9*	-1.0	-0.8
Ddah1	1.7*	1.8*	0.3	-0.1
Thbs1	1.2	1.5*	0.6	-0.1
Ctgf	1.4*	1.1*	0.2	0.1
Cyr61	1.2	1.2	-0.5	-0.3
Flna	0.5	0.5	-0.1	-0.3

c



Krebs, Supplementary Fig. 7

Fig. 2d

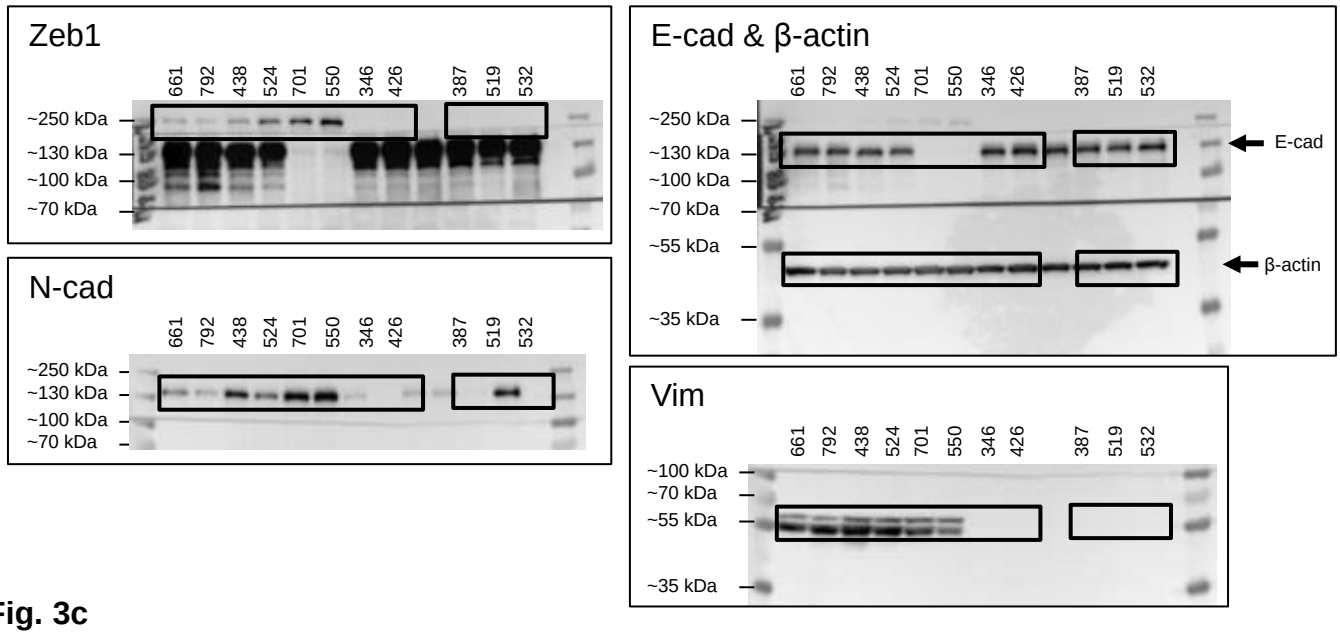


Fig. 3c

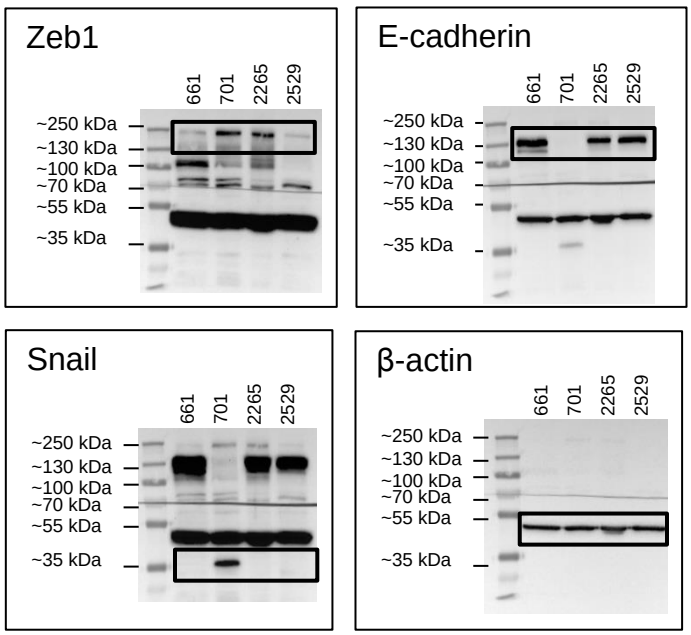


Fig. 3d

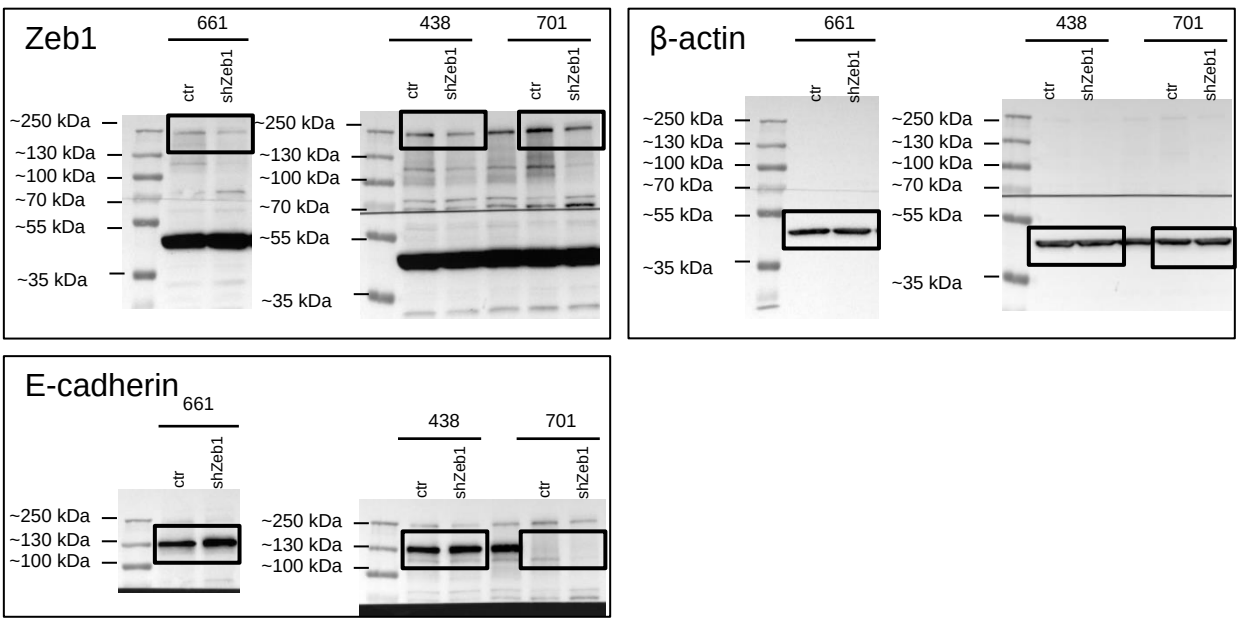


Fig. 3f

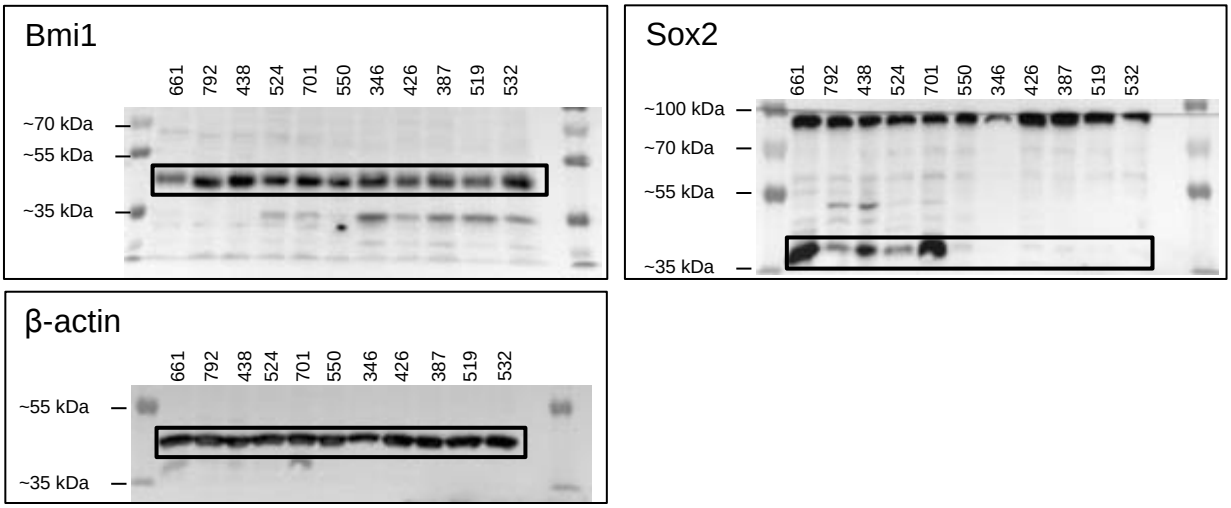


Fig. 3h

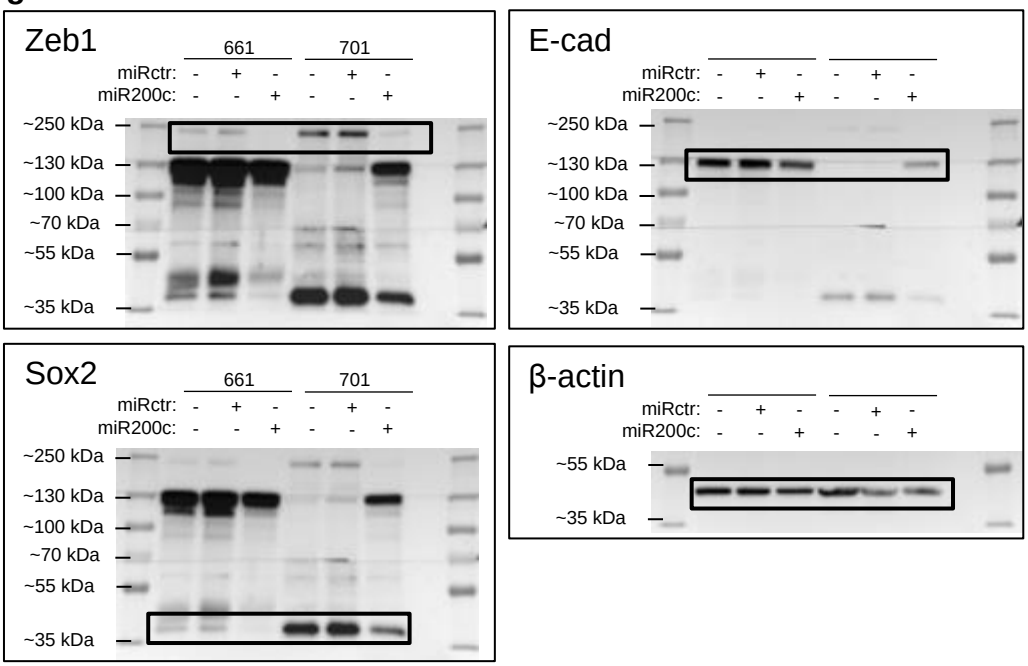


Fig. 5c

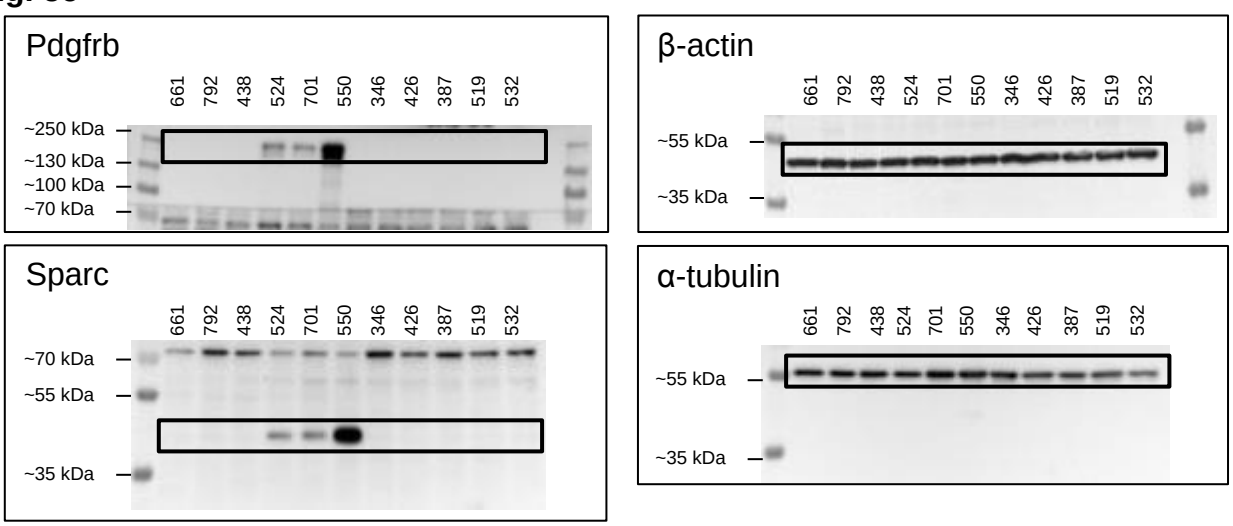
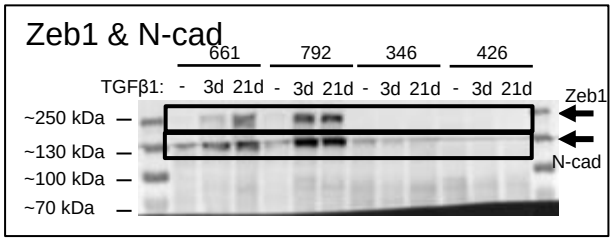
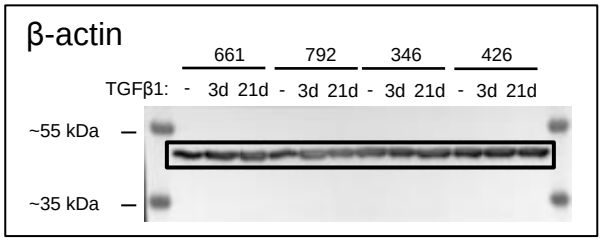


Fig. 6b


Zeb1 & N-cad
661
792
346
426
TGFβ1: - 3d 21d - 3d 21d - 3d 21d - 3d 21d
~250 kDa
~130 kDa
~100 kDa
~70 kDa
Zeb1
N-cad


β-actin
661
792
346
426
TGFβ1: - 3d 21d - 3d 21d - 3d 21d - 3d 21d
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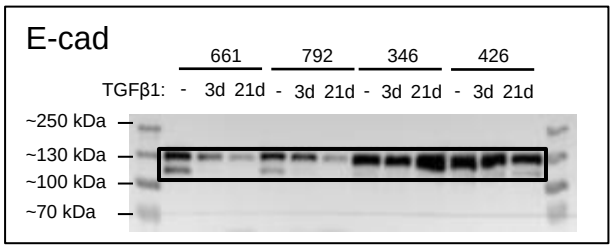
~35 kDa


E-cad
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792
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TGFβ1: - 3d 21d - 3d 21d - 3d 21d - 3d 21d
~250 kDa
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~70 kDa

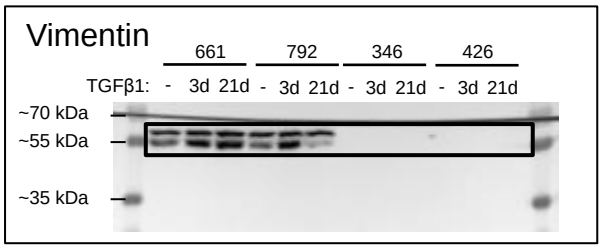
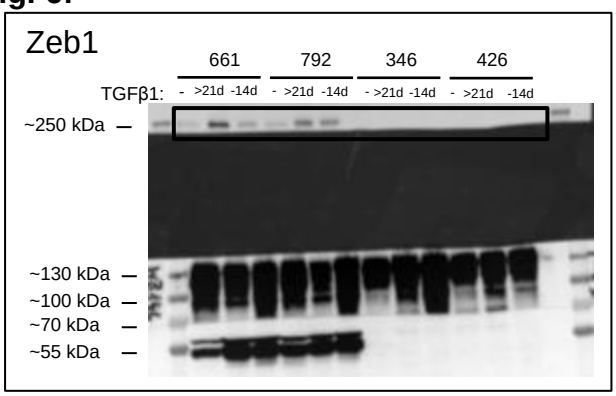

Vimentin
661
792
346
426
TGFβ1: - 3d 21d - 3d 21d - 3d 21d - 3d 21d
~70 kDa
~55 kDa
~35 kDa

Fig. 6f


Zeb1
661
792
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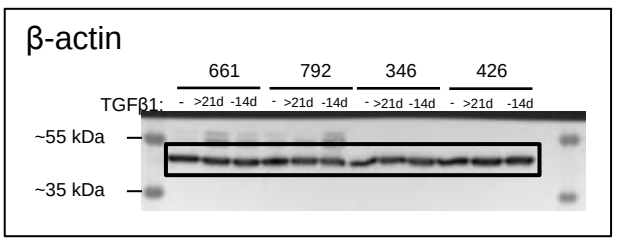
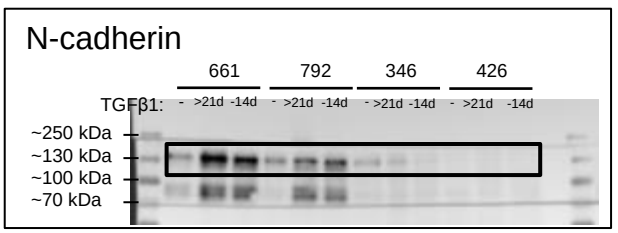
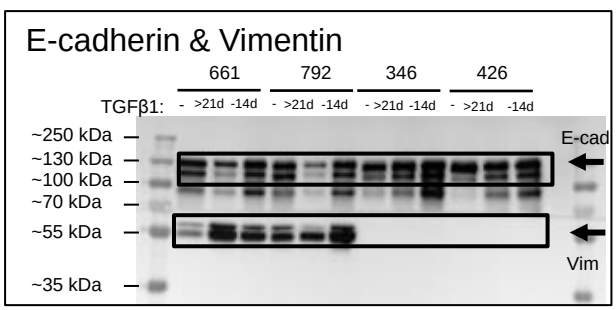
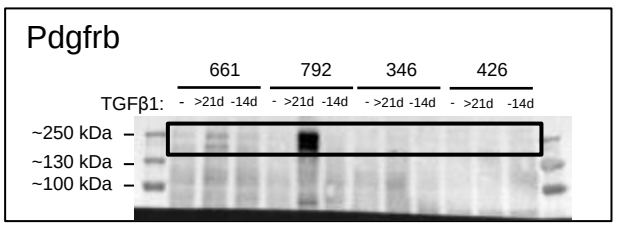
TGFβ1: - >21d -14d - >21d -14d - >21d -14d - >21d -14d
~250 kDa
~130 kDa
~100 kDa
~70 kDa
~55 kDa


β-actin
661
792
346
426
TGFβ1: - >21d -14d - >21d -14d - >21d -14d - >21d -14d
~55 kDa
~35 kDa


N-cadherin
661
792
346
426
TGFβ1: - >21d -14d - >21d -14d - >21d -14d - >21d -14d
~250 kDa
~130 kDa
~100 kDa
~70 kDa


E-cadherin & Vimentin
661
792
346
426
TGFβ1: - >21d -14d - >21d -14d - >21d -14d - >21d -14d
~250 kDa
~130 kDa
~100 kDa
~70 kDa
~55 kDa
~35 kDa
E-cad
Vim


Pdgfrb
661
792
346
426
TGFβ1: - >21d -14d - >21d -14d - >21d -14d - >21d -14d
~250 kDa
~130 kDa
~100 kDa